Case control study

Lack of association between estrogen receptor β dinucleotide repeat polymorphism and autoimmune thyroid diseases in Japanese patients

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Published: 31 January 2001

BMC Medical Genetics 2001, **2**:1

Received: 19 December 2000
Accepted: 31 January 2001

This article is available from: http://www.biomedcentral.com/1471-2350/2/1

Abstract

Background: The autoimmune thyroid diseases (AITDs), such as Graves' disease (GD) and Hashimoto's thyroiditis (HT), appear to develop as a result of complex interactions between predisposing genes and environmental triggers. Susceptibility to AITDs is conferred by genes in the human leukocyte antigen (HLA) and genes unlinked to HLA, including the CTLA-4 gene. Recently, estrogen receptor (ER) β, located at human chromosome 14q23-24.1, was identifed. We analyzed a dinucleotide (CA)n repeat polymorphism located in the flanking region of ERβ gene in patients with AITDs and in normal subjects. High heterozygosity makes this polymorphism a useful marker in the genetic study of disorders affecting female endocrine systems. We also correlated a ERβ gene microsatellite polymorphism with bone mineral density (BMD) in the distal radius and biochemical markers of bone turnover in patients with GD in remission.

Results: Fourteen different alleles were found in 133 patients with GD, 114 patients with HT, and 179 controls subjects. The various alleles were designated as allele*1 through allele*14 according to the number of the repeats, from 18 to 30. There was no significant difference in the distributions of ERβ alleles between patient groups and controls. Although recent study demonstrated a significant relation between a allele*9 in the ERβ gene and BMD in postmenopausal Japanese women, there were no statistically significant interaction between this allele and BMD in the distal radius, nor biochemical markers in patients with GD in remission.

Conclusions: The present results do not support an association between the ERβ microsatellite marker and AITD in the Japanese population. We also suggest that the ERβ microsatellite polymorphism has at most a minor pathogenic importance in predicting the risk of osteoporosis as a complication of GD.

Background

As outlined in a paper we have already published, we analyzed a dinucleotide (TA)n repeat polymorphism lying upstream of the human estrogen receptor (ER) α gene in patients with autoimmune thyroid diseases (AITDs) in normal subjects [1]. Seventeen different alleles were found in 130 patients with Graves' disease (GD), 93 patients with Hashimoto's thyroiditis (HT), and 190 control subjects [1]. There was no significant difference in the distributions of ERα alleles between patients and controls [1].

The pathogenesis of AITDs involves complex interactions between genetic and environmental factors [2,3]. Susceptibility to AITDs is conferred by genes in the human leukocyte antigen (HLA) [4,5,6,7,8] and genes unlinked to HLA, including the cytotoxic T lymphocyte antigen (CTLA)-4 gene [9]. The existence of an ERα gene polymorphism has been documented, and its association to some variant ERα genotypes found in breast cancer [10,11], hypertension [12], osteoporosis [13,14], generalized osteoarthritis [15], and some autoimmune diseases such as rheumatoid arthritis [16] has been reported.

A gene for a second type of human ER (ERβ) was recently identified [17]. The ERβ is located at human chromosome 14q23-24.1, where the aberration of DNA copy number in bone disorders is frequently found [18]. This locus was close to, but distinct from, a new susceptibility gene (GD-1) on chromosome 14q31 [19]. Recently, an association between some ERβ genotypes and osteoporosis has been reported [18]. ER belongs to the nuclear hormone receptor superfamily and modulates the transcription of target genes in response to estrogen [20], a potent immunomodulatory hormone [21]. Estrogens appear to play a central role in the immune response and immune-mediated diseases [21]. In view of the possible role of estrogens in the pathogenesis of AITDs, we analyzed a dinucleotide (CA)n repeat polymorphism located in the flanking region of ERβ gene [18] in patients with AITDs and in normal subjects. We also studied associaitons of a ERβ gene microsatellite polymorphism with distal radius bone mineral density (BMD) and biochemical markers of bone turnover to determine how this polymorphism might influence the development of osteoporosis as a complication of GD.

Results and discussion

Allele frequencies of ERβ gene microsatellite polymorphism in patients and controls

In general, estrogen action is thought to be mediated by ER, a member of the nuclear receptor superfamily [20]. Recent studies have shown the presence of ERs on cells involved in immune response, namely thymocyte, macrophages and endothelial cells [21]. Particular attention has been focused on the dose-dependent influence of estrogen on immune response, which appears to be related to the clinical symptoms of autoimmunity (i.e., the effects of pregnancy or oral contraceptive pills) [21]. The influence of estrogens on cytokine production by target cells, through interference with their transcriptional activity, has also been the focus of various studies [21]. The effect of estrogens on the expression of the proto-oncogenes and oncosuppressor genes involved in programmed cell death (apoptosis) might also be relevant to human autoimmunity; in particular, the uncontrolled synovial lining cell hyperplasia associated with rheumatoid arthritis and the prolonged T-cell survival in systemic lupus erythematosus may be important [21].

Table 1: Allele frequencies of the ERβ gene polymorphism in patients with AITDs and in control subjects

ERβ polymorphism	Graves' disease n=133, (266 alleles)	Hashimoto's thyroiditis n=114, (228 alleles)	Controls n=179, (358 alleles)
allele *1	8 (3.0%)	6 (2.6%)	11 (3.1%)
allele *2	0 (0%)	2 (0.9%)	1 (0.3%)
allele *3	0 (0%)	3 (1.3%)	1 (0.3%)
allele *4	6 (2.3%)	4 (1.8%)	14 (3.9%)
allele *5	57 (21.4%)	54 (23.7%)	87 (24.3%)
allele *6	20 (7.5%)	19 (8.3%)	24 (6.7%)
allele *7	15 (5.6%)	6 (2.6%)	21 (5.9%)
allele *8	32 (12.0%)	29 (12.7%)	36 (10.1%)
allele *9	18 (6.8%)	23 (10.1%)	32 (8.9%)
allele *10	74 (27.8%)	44 (19.3%)	87 (24.3%)
allele *11	27 (10.2%)	30 (13.2%)	31 (8.7%)
allele *12	9 (3.4%)	6 (2.6%)	7 (2.0%)
allele *13	0 (0%)	1 (0.4%)	5 (1.4%)
allele *14	0 (0%)	1 (0.4%)	1 (0.3%)

The number of subjects is shown with the corresponding percentage in parentheses.

The ERβ gene, isolated by Mosselman et al. [17], has 96% conserved amino acid residues in the DNA binding domain and 58% conserved residues in the ligand binding domain compared with the respective residues for ERα. The ERβ microsatellite polymorphism we analyzed in the present study has been recently characterized by Tsukamoto et al. [22]. High heterozygosity (0.93) makes this polymorphism a useful marker in the genetic study of disorders affecting female endocrine systems [22]. Indeed, an association of some ERβ genotypes with osteoporosis has been reported [18]. Thus, we investigated the relation between a dinucleotide (CA)n repeat polymorphism lying upstream of the human ER gene and AITDs.

Fourteen different alleles were found in 133 patients with GD, 114 patients with HT, and 179 controls subjects. The various alleles were designated as allele*1 through allele*14 according to their sizes, which ranged from 150 bp (containing 18 CA repeats with a 114 bp segment of amplified flanking sequences) to 176 bp (30 CA repeats). The distribution of ERβ alleles in the three groups is shown in Table 1. The allelic frequencies in our control population are in agreement with those previously published [18]. Allele frequencies in our GD patients and our control subjects did not differ significantly ($X^2 = 11.71$, 13 degrees of freedom, $P = 0.55$). Allele frequencies in our HT patients and our control subjects also did not differ significantly ($X^2 = 16.05$, 13 degrees of freedom, $P = 0.25$). According to our power calculations, we could exclude both associations with relative risk > 0.5 for the groups analyzed in Table 1 (GD patients vs. control subjects) and associations with relative risk > 0.3 for the groups analyzed in Table 1 (HT patients vs. control subjects).

Table 2: Comparison of background and biochemical data of GD patients in remission between the two groups of genotype

Items	Genotype		
	I (-)	I (+)	P value
Subjects	81	11	
Age	55.0 ± 11.1	59.1 ± 10.6	n.s
Radius BMD (Z-score)	0.104 ± 1.677	0.714 ± 1.313	n.s
S-bone type ALP (IU/L)	71.3 ± 45.0	74.2 ± 34.5	n.s.
S-osteocalcin (ng/mL)	6.3 ± 2.8	5.1 ± 2.3	n.s.
U-total hydroxyproline (nmol/mmol creatinine)	18.6 ± 8.8	19.9 ± 5.3	n.s.
U-total deoxypyridinoline (nmol/mmol creatinine)	5.0 ± 2.2	6.4 ± 3.4	n.s.

S, serum; U, urine; BMD, bone mineral density; ALP, alkaline phosphatase; n.s., not significant. Data are means ± SD except where noted.

Associations of ERβ gene microsatellite polymorphism with distal radius BMD and biochemical markers of bone turnover

Ogawa et al. [18] demonstrated that, when they separated postmenopausal Japanese women into two groups bearing at least one allele*9 (26 CA repeat) and those who did not, the former subjects had significantly higher Z score of L2-4 BMD. Thus, Z score of distal radius BMD and bone turnover markers in 92 female GD patients in remission were compared between the groups (Table 2). There was no significant difference of the Z score of distal radius BMD and bone turnover markers between the groups.

Conclusions

Our data did not appear to indicate any association between a dinucleotide repeat polymorphism located in the flanking region of ERβ gene and the AITDs analyzed. However, this does not rule out the ERβ gene as a whole; other polymorphisms within the gene could still be associated with AITD. There are also limitations in using microsatellite markers with multiple allleles in case-control studies. Our result might be indicative of a large diversity in the genetic backgrounds of AITDs, although this observation deserves further analysis in a larger group of AITD patients. We also suggest that the ERβ microsatellite polymorphism has at most a minor pathogenic importance in predicting the risk of osteoporosis as a complication of GD, although further study is warranted to confirm the affects of this polymorphism on BMD and risk of osteoporosis.

Materials and methods

Materials and methods used in this paper follow closely on the ones described in a paper we have already published [1].

Subjects

One hundred and thirty-three unrelated Japanese women with GD and 114 unrelated Japanese women with HT participated in this study. GD was diagnosed from clinical symptoms and biochemical confirmation of hyperthyroidism, including diffuse goiter, ophthalmopathy, elevated radioactive iodine uptake, and elevated thyroid hormone levels. HT patients had documented clinical and biochemical hypothyroidism requiring thyroid hormone replacement therapy and showed autoantibodies against thyroid peroxidase with or without antibodies against thyroglobulin. Remission of GD is defined as follows: both thyrotropin receptor antibody (TRAb) and thyroid-stimulating antibody (TSAb) in a negative range for 6 months, and a subsequent relatively stable euthyroid state over at least 6 months after cessation of antithyroid drug treatment. Remission ranged from 0.5 to 22.0 years. The effect of the ERβ microsatellite polymorphism on BMD in the distal radius and biochemical markers of bone turnover were investigated in the 92 GD patients optional in remission (age range, 47 to 78 years), none of whom had been treated with radioiodine, surgery, or drugs that could affect bone turnover or BMD prior to the study. One hundred and seventy-nine unrelated Japanese women without clinical evidence or family history of any autoimmune diseases were selected as normal control subjects. All study participants lived in the Tokyo metropolitan area. The research protocol was approved by the ethics committee of Showa University hospital, and informed consent was obtained from all patients and controls.

Measurement of biochemical markers

Blood and urine samples were collected early in the morning after an overnight fast. Both serum and urine were stored at -70°C until assayed. Serum concentration of bone alkaline phosphatase (B-ALP) was measured by densitometric scanning following polyacrylamide disc gel electrophoresis (PAGE). Osteocalcin (OC) was measured in serum with an IRMA. Materials for both assays were obtained from Mitsubishi Kagaku BCL (Tokyo, Japan). Intra- and interassay coefficients of variation were less than 10%. Urinary hydroxyproline (Hp) and deoxypyridinoline (Dpyr) were measured with high-performance liquid chromatography (HPLC; Japan Spectroscopic, Tokyo, Japan). Intra-assay coefficients of variation were less than 2.0% for Hp and 5.8% for Dpyr. Interassay coefficients of variation were less than 0.2% for Hp and 6.8% for Dpyr. Values of urinary Hp and Dpyr were corrected for urinary creatinine concentration, which was measured with an autoanalyzer.

Bone mineral density analysis

BMD was measured at the distal one third of the left radius with dual energy X-ray absorptiometry (DEXA; DTX-200, Osteometer, Copenhagen, Denmark). With this equipment, coefficients of variation in our laboratory were 1.0%. BMD was expressed as a Z-score representing the number of standard deviations (SDs) by which a value deviated from the mean BMD in 4095 healthy Japanese female controls aged 20 to 90 years.

Determination of microsatellite polymorphism by polymerase chain reaction

Genomic DNA was isolated from whole blood with a Genomix kit (Talent, Trieste, Italy). Microsatellite marker loci were typed with fluorescence-based methods [23,24]. Polymerase chain reaction (PCR) was performed with oligonucleotide primers designed to amplify a polymorphic (CA)n repeat in the flanking region of the human ERβ gene [18]. PCR was performed in a total volume of 20 μl of the following mixture: 100 ng of human genomic DNA, 5 pmol of each primer (Cy-5'-GGTAAACCATGGTCTGTACC-3' and 5'-AACAAAATGTTGAATGAGTGGG-3'), 200 μM of each dNTPs, 2 μl of 10 x reaction buffer (Takara Shuzo Co., Kyoto, Japan); and 1 unit of *Taq* DNA polymerase (Takara Shuzo Co.). Thirty PCR cycles of 30 sec at 94°C, 30 sec at 61°C, and 30 sec at 72°C were performed. The amplified fragments were analyzed by electrophoresis with an automatic DNA sequencer with 8% or 6% polyacrylamide gels containing 7 M Urea (Amersham Pharmacia Biotech, Buckingamshire, UK). The raw data were converted to dinucleotide repeat polymorphic band patterns with the use of a software program (Amersham Pharmacia Biotech). The number of CA repeats in each amplified product was determined by comparison with a ladder of control DNAs.

Statistical Analyses

Comparisons between the various alleles in patients with AITDs and in controls were made with the X^2- test. Fisher's exact test was used when necessary. The relative risk was calculated by Woolf's method [25]. Comparison of BMD in Z scores and biochemical markers between the group of individuals possessing one or two alleles of the genotype and the group without that genotype were performed using Student's *t* test. A *P* value less than 0.05 was considered statistically significant.

Acknowledgements

This work was supported in part by the High-Technology Research Center Project from the Ministry of Education, Science, Sports and Culture of Japan.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/content/backmatter/1471-2350-2-1-b1.pdf